Available online at www.sciencedirect.com
SCIENCE DIRECT®
Pesticide Biochemistry and Physiology 81 (2005) 145–153
PESTICIDE
Biochemistry & Physiology
www.elsevier.com/locate/ypest

Toxicological and biochemical characterization of coumaphos resistance in the San Roman strain of *Boophilus microplus* (Acari: Ixodidae)☆

Andrew Y. Li[a,*], John H. Pruett[a], Ronald B. Davey[b], John E. George[a]

[a] *USDA, ARS, Knipling-Bushland US Livestock Insects Research Laboratory, 2700 Fredericksburg Road, Kerrville, TX 78028, USA*
[b] *USDA, ARS, Cattle Fever Tick Research Laboratory, 22675 N. Moorefield Road, Edinburg, TX 78541, USA*

Received 25 October 2004; accepted 3 December 2004

Abstract

The San Roman strain of the southern cattle tick, *Boophilus microplus*, collected from Mexico was previously reported to have a high level of resistance to the organophosphate acaricide coumaphos. An oxidative detoxification mechanism was suspected to contribute to coumaphos resistance in this tick strain, as coumaphos bioassay with piperonyl butoxide (PBO) on larvae of this resistant strain resulted in enhanced coumaphos toxicity, while coumaphos assays with PBO resulted in reduced toxicity of coumaphos in a susceptible reference strain. In this study, we further analyzed the mechanism of oxidative metabolic detoxification with synergist bioassays of coroxon, the toxic metabolite of coumaphos, and the mechanism of target-site insensitivity with acetylcholinesterase (AChE) inhibition kinetics assays. Bioassays of coroxon with PBO resulted in synergism of coroxon toxicity in both the San Roman and the susceptible reference strains. The synergism ratio of PBO on coroxon in the resistant strain was 4.5 times that of the susceptible strain. The results suggested that the *cytP450*-based metabolic detoxification existed in both resistant and susceptible strains, but its activity was significantly enhanced in the resistant strain. Comparisons of AChE activity and inhibition kinetics by coroxon in both susceptible and resistant strains revealed that the resistant San Roman strain had an insensitive AChE, with a reduced phosphorylation rate, resulting in a reduced bimolecular reaction constant. These data indicate a mechanism of coumaphos resistance in the San Roman strain that involves both insensitive AChE and enhanced *cytP450*-based metabolic detoxification.
Published by Elsevier Inc.

Keywords: Coumaphos; Organophosphate; Mechanism of resistance; AChE; *cytP450s*; Cattle tick; *Boophilus microplus*

1. Introduction

The southern cattle tick, *Boophilus microplus* (Canestrini), is a serious ectoparasite of cattle in

☆ This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

* Corresponding author. Fax: +1 830 792 0337.
E-mail address: Andrew.Li@ars.usda.gov (A.Y. Li).

0048-3575/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.pestbp.2004.12.002

many tropical and subtropical regions of the world, and a vector of *Babesia bovis* and *B. bigemina*, the causative protozoan agents of bovine babesiosis [1]. An intensive national cattle tick eradication campaign was initiated in 1906 to eliminate *B. microplus* along with the cattle tick *Boophilus annulatus* (Say) from the US through quarantine, systematic acaricide treatment of infested animals, and pasture vacation [2]. *B. microplus* was successfully eradicated from the continental US in 1961, except in a few counties in south Texas along the US–Mexican border [2,3]. Coumaphos (CoRal), an organophosphate (OP) acaricide, has been used in the United States since 1968, exclusively in recent years, by the USDA, US Cattle Fever Tick Eradication Program (CFTEP) to prevent the reintroduction of *Boophilus* ticks from Mexico through the importation of cattle [2,4]. Coumaphos is also used to eradicate infestation of *Boophilus* ticks established in south Texas when tick-infested cattle, horse and ungulate wildlife from Mexico cross the Rio Grande river into a quarantined area regulated by the CFTEP.

Boophilus microplus is a one-host tick known to have the capacity to develop resistance to various acaricides [5–7]. Due to its high toxicity to ticks and low toxicity to cattle, coumaphos is the only acaricide registered for use in the importation dipping vats and to eradicate *Boophilus* tick outbreaks in south Texas, thus playing a pivotal role in preventing *B. microplus* along with *B. annulatus* from reinfesting the US [6]. The development of resistance to coumaphos and other OP acaricides in Mexican strains of *B. microplus* poses a major threat to the continued success of the CFTEP [5,8,9]. To develop new tick eradication and control strategies to overcome the acaricide resistance problem, it is critical to understand the mechanisms involved in resistance. Elucidation of resistance mechanisms to OPs, particularly to coumaphos in *B. microplus* from Mexico would also have great value in the development of rapid molecular and biochemical resistance detection techniques.

Li et al. [6] determined the levels of resistance to coumaphos in various strains of *B. microplus* collected from Mexico using a slightly modified version of the Food and Agriculture Organization (FAO)'s larval packet test (LPT). The San Roman strain was found to be the most resistant strain, with a resistance ratio at 10.1. Results of synergist bioassays suggest the existence of an oxidative detoxification mechanism in several OP-resistant tick strains, including the San Roman strain [6]. However, the details of such oxidative detoxification mechanism in *B. microplus* remain unclear. OPs act by inhibiting acetylcholinesterase (AChE), the key enzyme for nervous system function in arthropods [10]. It has been demonstrated in insects that insensitive AChE is a major mechanism of resistance to OPs [11]. Insensitive AChE as a mechanism of resistance to OPs in *B. microplus* was first suggested by Lee and Bantham [12], and was later reported in the OP-resistant Tuxpan strain of *B. microplus* from Mexico [13]. Insensitive AChE mechanism in *B. microplus* was also confirmed in a recent study by Pruett [14] in more tick strains from Mexico, including the San Roman strain. The diethyl-OP paraoxon was used as a substrate of AChE in this study. Since coumaphos is a major OP acaricide used for tick control in Mexico, the biochemical mechanisms of coumaphos resistance in ticks from Mexico can be better characterized by testing metabolic detoxification of coroxon, the active metabolite of coumaphos, and by using coroxon as the substrate of AChE in enzyme inhibition study.

This paper reports the results of coroxon synergist bioassays and inhibition kinetics of AChE by coroxon that further analyze the mechanisms of oxidative metabolic detoxification and target-site insensitivity for coumaphos resistance in the San Roman strain of *B. microplus*.

2. Materials and methods

2.1. Tick strains

Two strains of *B. microplus* were used in this study. The coumaphos-resistant San Roman strain was collected from a ranch near Champoton, Campeche, Mexico in August of 1998. The Gonzalez strain was obtained from an outbreak of ticks in Zapata County, Texas in 1994. The Gonzalez

strain was determined to be susceptible to all major classes of acaricides, and therefore, was used as a reference strain. Both strains have been maintained on cattle at the USDA ARS Cattle Fever Tick Research Laboratory (CFTRL) in Mission, Texas since their collection. The procedures for rearing ticks on bovine hosts and maintaining the non-parasitic stages in the laboratory were described elsewhere [15]. In comparison to the susceptible reference tick strain, the San Roman strain had an 8-fold resistance upon arrival at CFTRL, and has been challenged with 0.2–0.4% coumaphos in the following generations to increase and maintain resistance.

2.2. Chemicals

Technical grade coumaphos (97.4% a.i.) was obtained from BayVet (Shawnee, KS). Technical grade coroxon (99.5% a.i.) was obtained from the repository of the US Environmental Protection Agency (Atlanta, GA). Piperonyl butoxide (PBO), an inhibitor of cytochrome P450s (*cytP450s*), was purchased from Aldrich, (Milwaukee, WI). Trichloroethylene and olive oil were purchased from Sigma (St. Louis, MO).

2.3. Bioassay

Toxicity of coumaphos and coroxon and the effect of PBO on the toxicity of those two compounds in both the Gonzalez and the San Roman strains were determined by the FAO larval packet test described elsewhere [6,16]. Briefly, technical grade coumaphos or coroxon was dissolved in trichloroethylene to make a stock solution, which was then diluted to generate a top dose in diluent containing two parts of trichloroethylene and one part of olive oil. The top dose was serially diluted to generate at least five testing doses. PBO was added at a constant rate of 1% to coumaphos or coroxon dilutions to test its synergistic effect. A Whatman #1 filter paper (7.5 × 9.0 cm; Whatman, Maidstone, Kent, UK) was treated with 1 ml testing solution, and the solvent was allowed to evaporate for 2 h before being folded into a packet. Approximately one hundred 12- to 16-day-old larvae were added into each packet and the sides of the packet were sealed with bulldog clips. Three replicates were prepared for each dose. The packets were placed in an environmental chamber (27 °C, 90% RH, photoperiod = 12:12L:D) for 24 h before the numbers of live and dead larvae were counted.

2.4. Enzyme extraction

Enzyme extracts were prepared by grinding, in a glass homogenizer, 0.1 g of 14-day-old tick larvae of both the Gonzalez (generation 30) and the San Roman (generation 18) strains in 1 ml of tick extraction buffer (TEB; 10 mM $NaPO_4$, pH 6.5, containing 20% sucrose, 1 mM EDTA, and 0.5% Triton X-100). Enzyme was extracted for 2 h at 4 °C with gentle agitation. Homogenates were centrifuged (Hermle Z 360 K) at 14,400*g* for 15 min at 4 °C and the solubilized proteins collected in the supernatant. The protein concentration of each extract was determined with a micro-BCA method using bovine serum albumin as a standard (Pierce, Rockford, IL).

2.5. Determination of AChE kinetic parameters

A modified Ellman assay [17], conducted in microplates, was used to measure AChE activity by determining the maximum velocity (V_{max}) and the Michaelis constant (K_m) for each tick extract [14]. Briefly, 16 µl containing 20 µg of extracted tick proteins (six replicates) was added to 200 µl of 50 mM sodium phosphate buffer, pH 7.5, containing the substrate acetylthiocholine iodide (ASCh, in double dilutions from 120×10^{-6} to 3.75×10^{-6} M) and 0.32 M Ellman's reagent (5,5′-dithio-bis)2-nitrobenzoic acid; DTNB, Sigma Chemical, St. Louis, MO). Kinetic constants (V_{max} and K_m) were calculated with a rigorous nonlinear least squares method (Enzyme Kinetics Pro, ChemSW Software) with the mean initial velocity (V_o) ± SD of replicates for each substrate concentration.

2.6. Coroxon inhibition of AChE activity

A modification of the methods of Chen et al. [18] was used to measure the kinetic parameters

of AChE inhibition by coroxon, the oxon form of coumaphous. The rate of AChE inhibition was measured in the presence of 1.2×10^{-4} M ASCh according to the modified Ellman assay described above with 20 μg of enzyme extract (six replicates), and coroxon concentrations of 3.5×10^{-5}, 3.0×10^{-5}, 2.5×10^{-5}, 2.0×10^{-5}, 1.5×10^{-5}, and 1.0×10^{-5} M for the San Roman extracted AChE and 2.0×10^{-5}, 1.5×10^{-5}, 1.25×10^{-5}, 1.0×10^{-5}, 0.75×10^{-5}, and 0.5×10^{-5} M for the Gonzalez extracted AChE. The progressive inhibition of AChE at each concentration of coroxon was monitored over time (12 min, readings at 2 min intervals) as described by Pruett [14] as a modification of the method of Chen et al. [18], and the percent residual AChE activity relative to an uninhibited control was calculated. The bimolecular reaction constant (k_i), the dissociation constant (K_d), and the phosphorylation constant (k_2), were calculated according to the method described by Pruett [14] as a modification of the method of Chen et al. [18].

2.7. Data analysis

POLO-PC [19] was used to analyze dose–mortality response of all bioassays to generate probit regression data. Resistance ratio (RR) to coumaphos or coroxon of the San Roman strain was calculated relative to the Gonzalez strain, and PBO synergism ratio (SR) was calculated relative to the bioassay containing only coumaphos or coroxon for the same strain. RRs and SRs were calculated using the method of Robertson and Preisler [20] for non-parallel probit lines. Statistical analyses of the kinetic parameters were made with the Student's t test if the data were normally distributed or with the Mann–Whitney rank sum test if the data failed a test of normality (SigmaStat software [21]).

3. Results

3.1. Effects of PBO on coumaphos and coroxon toxicity

The results of probit analysis of dose–mortality responses of the Gonzalez and San Roman strains of *B. microplus* to coumaphos and coroxon, with and without PBO, are summarized in Table 1. In comparison to the susceptible Gonzalez strain, the San Roman strain demonstrated a 10.09-fold resistance to coumaphos and a 22.60-fold resistance to coroxon. PBO had opposite effects on coumaphos toxicity in the resistant (San Roman) and susceptible (Gonzalez) strains. PBO synergized coumaphos toxicity in coumaphos-resistant San Roman strain (SR = 3.08), while it inhibited coumaphos toxicity in the susceptible Gonzalez

Table 1
Summary of dose–mortality responses to coumaphos and coroxon with and without PBO in susceptible and resistant strains of *B. microplus*[a]

	n	Slope (SE)	LC_{50} (95% CI)[b]	RR[c] (95% CI)	SR[d] (95% CI)
Gonzalez					
Coumaphos	2248	9.88 (0.81)	0.08236 (0.07083–0.08944)		
Coumaphos + PBO	1747	7.87 (0.36)	0.11106 (0.10210–0.12245)		0.74 (0.71–0.77)
Coroxon	2093	3.83 (0.16)	0.00180 (0.00154–0.00217)		
Coroxon + PBO	2600	6.38 (0.39)	0.00057 (0.00049–0.00063)		3.18 (2.98–3.39)
Sam Roman					
Coumaphos	1861	7.09 (0.35)	0.82716 (0.77976–0.87325)	10.09 (9.68–10.52)	
Coumaphos + PBO	2586	5.22 (0.20)	0.26774 (0.24060–0.29568)		3.08 (2.96–3.23)
Coroxon	1452	9.90 (0.53)	0.04071 (0.03744–0.04412)	22.60 (21.40–23.86)	
Coroxon + PBO	2060	3.86 (0.20)	0.00286 (0.00237–0.00326)		14.26 (13.37–15.20)

[a] Results of coumaphos bioassays have appeared previously in Li et al. [6].
[b] CI, confidence interval. All values are % a.i.
[c] RR, resistance ratio.
[d] SR, synergism ratio.

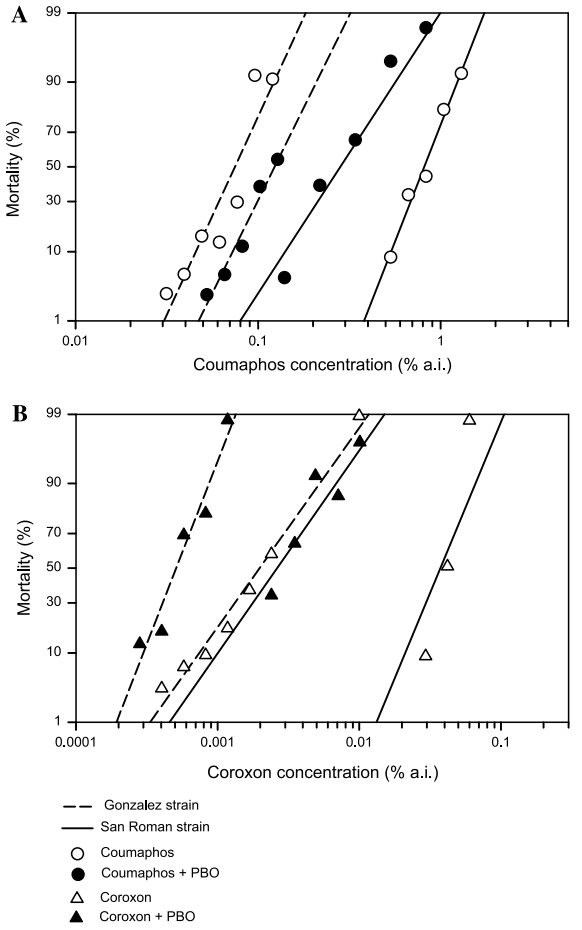

Fig. 1. Comparisons of effect of PBO on toxicity of coumaphos (A) and coroxon (B) in a coumaphos-resistant (San Roman) strain and a susceptible (Gonzalez) reference strain. Each data point represents the mean of three replicates.

strain (SR = 0.74; Table 1, Fig. 1A). PBO synergized coroxon toxicity in both the Gonzalez and the San Roman strains with SRs of 3.18 and 14.26, respectively (Table 1; Fig. 1B).

3.2. AChE activity and inhibition kinetics

The V_{max} value of the San Roman strain AChE ($V_{max} = 0.67 \times 10^{-4}$ mol/min/mg larval protein) was not statistically different from that of the Gonzalez strain AChE ($V_{max} = 1.06 \times 10^{-4}$ mol/min/mg larval protein) ($t = 1.92$, df = 10, $P = 0.084$). However, the K_m value for the San Roman strain AChE ($K_m = 8.40 \times 10^{-6}$ M) was significantly lower than the K_m for the Gonzalez strain AChE ($K_m = 13.96 \times 10^{-6}$ M; $t = 15.25$, df = 10, $P = 0.001$), indicating an increased affinity of San Roman AChE for the substrate. Insensitivity of the San Roman strain AChE to coroxon inhibition was demonstrated in the measurement of experimentally determined parameters of inhibition kinetics. The San Roman strain AChE exhibited a greater affinity for coroxon as evidenced by a lower dissociation constant (K_d) value (median $= 7.65 \times 10^{-6}$ M) than that of the Gonzalez AChE (median $= 13.08 \times 10^{-6}$ M; $t = 22.0$,

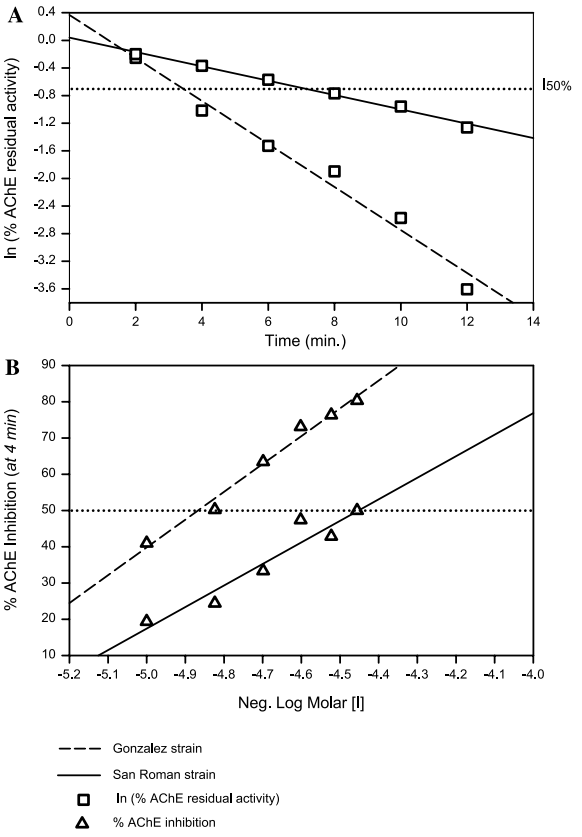

Fig. 2. Results of AChE inhibition kinetics. (A) Comparison of change of % AChE residual activity over time between the San Roman and Gonzalez strains. A constant coroxon concentration of 2.0×10^{-5} molar was used for this test. (B) Comparison of % AChE inhibition of by various concentration of coroxon at 4 min between the San Roman and Gonzalez strains. Each data point represents the mean of six replicates.

df = 6, $P = 0.004$). The rate of AChE phosphorylation by coroxon, as measured by the phosphorylation constant, was significantly slower for San Roman (median $k_2 = 0.804\ \text{min}^{-1}$) than that of the Gonzalez AChE (median $k_2 = 2.34\ \text{min}^{-1}$; $t = 21.0$, df = 6, $P = 0.002$). The slower rate of phosphorylation by the San Roman AChE resulted in a significantly lower value for the bimolecular reaction constant ($k_i = 1.03 \times 10^{-5}\ \text{M}^{-1}\ \text{min}^{-1}$) than that obtained for the Gonzalez AChE ($k_i = 1.76 \times 10^{-5}\ \text{M}^{-1}\ \text{min}^{-1}$; $t = -31.27$, df = 10, $P < 0.001$).

The slower inhibition rate for San Roman AChE is illustrated in Fig. 2. The time to 50% inhibition of AChE activity in the presence of 2.0×10^{-5} M coroxon was shorter for the Gonzalez strain AChE (3.45 min) and longer for the San Roman strain AChE (7.05 min, Fig. 2A). Gonzalez strain AChE activity was inhibited to 50% of activity by exposure to coroxon at a concentration of 1.39×10^{-5} M for 4 min. Twice the amount of coroxon was required to inhibit the San Roman strain AChE to 50% of activity (3.53×10^{-5} M) in 4 min (Fig. 2B).

4. Discussion

Insensitive AChE and metabolic detoxification mechanisms of resistance to OPs have been demonstrated in many insect species [11,22]. Insensitive AChE has been considered the principle mechanism of resistance to OP acaricides in *B. microplus*, although metabolic detoxification mechanisms were also implicated [12–14,23–25]. Insensitive AChE as a mechanism of resistance is usually accompanied by either an increase in dissociation constant (K_d), reflecting a reduction of the affinity of AChE for the pesticide, or a decrease in the phosphorylation rate (K_2) [22]. Alteration of San Roman strain AChE activity relative to the susceptible strain Gonzalez AChE activity is evidenced by reduced activity (V_{max}), and increased affinity for substrate (decreased K_m). Although the affinity of AChE for coroxon, the substrate in this study, was increased in the San Roman strain in comparison to the Gonzalez strain, the reduced values of K_2 and K_i are indicative of a slower rate of enzyme phosphorylation by coroxon, yielding an AChE insensitive to the toxic effect of coumaphos. Similar reductions in V_{max} and K_i were also observed in the San Roman strain when a different substrate (paraoxon) was used in AChE inhibition assays [14]. The differences in the rate of inhibition between the resistant San Roman strain and the susceptible Gonzalez strain observed in this study indicate that the resistance to coumaphos, and possibly other OPs, demonstrated by the San Roman strain is at least partially due to insensitive AChE.

Although insensitive AChE has been demonstrated with in vitro enzyme kinetic assays in many OP-resistant pests, our understanding of the molecular basis for the altered AChE is very limited [11]. Genes encoding AChE have been sequenced in a number of insect species, and point mutations of AChE genes that are associated with OP resistance have been identified only in a few insect species including *Drosophila melanogaster*, and the Colorado potato beetle, *Leptinotarsa decemlineata* [10,22,26,27]. Although results of other investigations on AChE inhibition kinetics provided clear evidence for altered kinetic properties of AChE in OP-resistant strains of *B. microplus* [13,14], no point mutations associated with OP resistance were found in any of three identified and sequenced putative *B. microplus* AChE genes [28–31]. Polymorphism of AChE-encoding genes and the lack of point mutations associated with OP resistance also exist in insect species [10]. It has been suggested that alteration of AChE confirmation may occur post-translationally, and such post-translational modification could lead to insensitive AChE [10,28]. OP resistance may also be conferred by increased AChE gene expression that is unrelated to gene amplification, as demonstrated in an OP-resistant strain of the greenbug [32].

Desulfuration of the phophorothionate coumaphos to the more toxic oxon form (coroxon) is metabolically facilitated by microsomal oxidases, such as *cytP450s* [11,33]. PBO is an inhibitor of *cytP450s*, and thus inhibits the bioactivation of coumaphos. As expected, PBO reduced coumaphos toxicity in the Gonzalez strain, however, PBO unexpectedly increased coumaphos toxicity

in the San Roman strain, yielding a synergism ratio of 3.08. This suggests that, in addition to an oxidative mechanism that bioactivates coumaphos, the San Roman strain also possesses a significant oxidative detoxification mechanism. A *cytP450*-based oxidative detoxification mechanism is further supported by bioassays with coroxon, the active metabolite of coumaphos, in which PBO synergized coroxon toxicity at a rate 4.5 times higher in the San Roman strain (SR = 14.26) than in the Gonzalez strain (SR = 3.18). The fact that PBO also synergized coroxon toxicity in the Gonzalez strain perhaps indicates the existence of an intrinsic low level *cytP450*-based oxidative detoxification mechanism in the susceptible Gonzalez strain.

It is known that *cytP450s* exist in multiple isoforms within any organism, including arthropod species, and each isoform has a potentially narrow substrate specificity [34]. The role of *cytP450*-based oxidative detoxification has been documented as a major metabolic mechanism of resistance to pyrethroids in many insect species, such as housefly, mosquitoes, and the horn fly [35–37]. It has also been demonstrated in several insect species that *cytP450s*-mediated metabolic detoxification was responsible for resistance to OPs [38–41]. The substrate-specific nature of such *cytP450s*-mediated detoxification mechanism has been demonstrated in insects [41,42], and has also been implicated in *B. microplus* [6].

The coexistence of the *cytP450*-based metabolic detoxification and insensitive AChE mechanisms in the San Roman strain of *B. microplus* highlights the complex nature of coumaphos resistance. It is possible the synergistic interaction of these two different resistance mechanisms that accounts for the highest level of resistance to coumaphos demonstrated in this tick strain. The finding of the contribution of the *cytP450*-based metabolic detoxification mechanism in coumaphos resistance in *B. microplus* has important implications for the successful elimination of resistant ticks for the USDA's CFTEP, as well as successful control of coumaphos-resistant ticks in Mexico. The role of *cytP450*-mediated metabolic detoxification has been well defined in flies, and PBO has been used successfully as a synergist of pyrethroid insecticides to control pyrethroid-resistant flies [35,37,43]. But, it may not be feasible to use PBO as a synergist of coumaphos to control OP-resistant ticks, as PBO inhibits the bioactivation of coumaphos in susceptible ticks.

Acknowledgments

We thank Dave Krska and Brenda Burkett for their technical assistance in conducting the pesticide bioassays and the AChE kinetic assays, respectively, and Drs. Kun Yan Zhu and Pia Untalan for reviewing the manuscript.

References

[1] K.T. Friedhoff, R.D. Smith, Transmission of *Babesia* by ticks, in: M. Ristic, J.P. Kreier (Eds.), Babesiosis, Academic Press, New York, 1981, pp. 267–321.

[2] O.H. Graham, J.L. Hourrigan, Eradication programs for the arthropod parasites of livestock, J. Med. Entomol. 6 (1977) 629–658.

[3] R.A. Bram, J.E. George, Introduction of nonindigenous arthropod pests of animals, J. Med. Entomol. 37 (2000) 1–8.

[4] J.E. George, The campaign to keep *Boophilus* ticks out of the United States: technical problems and solutions, in: Proceedings of the 100th Annual Meeting of the US Animal Health Association, Spectrum, Richmond, VA, 1996, pp. 196–206.

[5] V.M. Santamaría, C.N. Soberanes, N.A. Ortiz, S.H. Fragoso, M.J. Osorio, I.F. Martínez, B.L. Franco, V.G. Delabra, D.R. Quezada, H.I. Giles, E.M. Ortiz, Analisis de la situacion Actual ediante el monitoreo de susceptibilidad a ixodicidas en *Boophilus microplus* de 1993 a 1999 y medidas preventivas para retardar la resistencia al amitraz en Mexico, in: IV Seminario Internacional de Parasitologia Animal: Control de la Resistancia en Garrapatas y Moscas de Importancia Veterinaria y Enfermedades que transmiten, 20–22 de Octubre 1999, Puerto Vallarta, Jalisco, Mexico, 1999, pp. 103–117.

[6] A.Y. Li, R.B. Davey, R.J. Miller, J.E. George, Resistance to coumaphos and diazinon in *Boophilus microplus* (Acari: Ixodidae) and evidence for the involvement of an oxidative detoxification mechanism, J. Med. Entomol. 40 (2003) 482–490.

[7] A.Y. Li, R.B. Davey, R.J. Miller, J.E. George, Detection and characterization of amitraz resistance in the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae), J. Med. Entomol. 41 (2004) 193–200.

[8] E.M. Ortiz, V.M. Santamaría, N.A. Ortiz, E.J. Osorio, C.N. Soberanes, B.R. Franco, R.D. Quezada, and S.H.

Fragoso, Hallazgos de resistencia en garrapatas *B. microplus* hacia ixodicidas piretroides en la Republica Mexicana. in: Proceedings of XIV Pan American Congress on Veterinary Science. Acapulco, Mexico, 1994, pp. 23–25.

[9] R.B. Davey, J.E. George, Efficacy of coumaphos applied as a dip for control of an organophosphate-resistant strain of *Boophilus microplus* (Acari: Ixodidae) on cattle, J. Econ. Entomol. 92 (1999) 1384–1389.

[10] F. Villatte, T.T. Bachmann, How many genes encode cholinesterase in arthropods?, Pestic. Biochem. Physiol. 73 (2002) 122–129.

[11] B.D. Siegfried, M.E. Scharf, Mechanisms of organophosphate resistance in insects, in: I. Ishaaya (Ed.), Biochemical Sites of Insecticide Action and Resistance, Springer-Verlag, Berlin, Germany, 2001, pp. 269–287.

[12] R.M. Lee, P. Bantham, The activity and organophosphate inhibition of cholinesterase from susceptible and resistant ticks (Acari), Entomol. Exp. Appl. 9 (1966) 13–24.

[13] F.C. Wright, E.H. Ahrens, Cholinesterase insensitivity: a mechanism of resistance in Mexican strains of *Boophilus microplus* (Acari: Ixodidae) against coumaphos, J. Med. Entomol. 25 (1988) 234–239.

[14] J.H. Pruett, Comparative inhibition kinetics for acetylcholinesterases extracted from organophosphate resistant and susceptible strains of *Boophilus microplus* (Acari: Ixodidae), J. Econ. Entomol. 95 (2002) 1239–1244.

[15] R.B. Davey, J. Garza Jr., G.D. Thompson, R.O. Drummond, Ovisopistional biology of the cattle tick, *Boophilus microplus* (Acari: Ixodidae), in the laboratory, J. Med. Entomol. 17 (1980) 287–289.

[16] R.J. Miller, R.B. Davey, J.E. George, Characterization of pyrethroid resistance and susceptibility to coumaphos in Mexican *Boophilus microplus* (Acari: Ixodidae), J. Med. Entomol. 36 (1999) 533–538.

[17] G.L. Ellman, K.D. Courtney, V. Andres Jr., R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.

[18] Z. Chen, R. Newcomb, E. Forbes, J. McKenzie, P. Batterham, The acetylcholinesterase gene and organophosphorus resistance in the Australian sheep blowfly, *Lucilla cuprina*, Insect Biochem. Mol. Biol. 31 (2001) 805–816.

[19] LeOra Software, A User's Guide to Probit or Logit Analysis, LeOra Software, Berkely, CA, 1987.

[20] J.L. Robertson, H.K. Preisler, Pesticide Bioassays with Arthropods, CRC, Boca Raton, FL, 1992.

[21] J. Kuo, E. Fox, S. McDonald, SigmaStat Statistical Software for Working Scientists, Jandel Scientific, San Rafael, CA, 1992.

[22] D. Fournier, A. Mutero, Modification of acetylcholinesterases as a mechanism of resistance to insecticides, Comp. Biochem. Physiol. 108C (1994) 19–31.

[23] W.J. Roulston, H.J. Schnitzerling, C.A. Schuntner, J.T. Wilson, Acetylcholinesterase insensitivity in the Biarra strain of the cattle tick *Boophilus microplus*, as a cause of resistance to organophosphorus and carbamate acaricides, Aust. J. Biosci. 21 (1968) 759–767.

[24] W.J. Roulston, C.A. Schuntner, H.J. Schnitzerlling, J.T. Wilson, Detoxification as a mechanism of resistance in a strain of the cattle tick *Boophilus microplus* (Canestrini) resistant to organophosphorus and carbamate compounds, Aust. J. Biosci. 22 (1969) 1585–1589.

[25] M.A. Villarino, G.G. Vagner, J.E. George, In vitro detection of acaricide resistance in *Boophilus microplus* (Acari: Ixodidae), Exp. Appl. Acarol. 28 (2002) 265–271.

[26] A. Mutero, M. Pralavorio, J.M. Bride, D. Fournier, Resistance-associated point mutations in insecticide insensitive acetylcholinesterase, Proc. Natl. Acad. Sci. USA 91 (1994) 5922–5926.

[27] K.Y. Zhu, J.M. Clark, Validation of a point mutation of acetylcholinesterase in Colorado potato beetle by polymerase chain reaction coupled with enzyme inhibition assay, Pestic. Biochem. Physiol. 57 (1997) 28–35.

[28] G.D. Baxter, S.C. Barker, Acetylcholinesterase cDNA of the cattle tick, *Boophilus microplus*: characterization and role in organophosphate resistance, Insect Biochem. Mol. Biol. 28 (1998) 581–589.

[29] G.D. Baxter, S.C. Barker, Analysis of the sequence and expression of a second putative acetylcholinesterase cDNA from organophosphate-susceptible and organophosphate-resistant cattle ticks, Insect Biochem. Mol. 32 (2002) 815–820.

[30] R. Hernandez, H. He, A.C. Chen, G.W. Ivie, J.E. George, G.G. Wagner, Cloning and sequencing of a putative acetylcholinesterase cDNA from *Boophilus microplus* (Acari: Ixodidae), J. Med. Entomol. 36 (1999) 766–770.

[31] K.B. Temeyer, R.B. Davey, A.C. Chen, Identification of a third *Boophilus microplus* (Acari: Ixodidae) cDNA presumptively encoding an acetylcholinesterase, J. Med. Entomol. 41 (2004) 259–268.

[32] J.-R. Gao, K.Y. Zhu, Increased expression of an acetylcholinesterase gene may confer organophosphate resistance in the greenbug, *Schizaphis graminum* (Homoptera: Aphididae), Pestic. Biochem. Physiol. 73 (2002) 164–173.

[33] R.D. O'Brien, in: Insecticides Action and Metabolism, Academic Press, NY, 1967, p. 332.

[34] J.G. Scott, Z. Wen, Cytochromes P450 of insects: the tip of the iceberg, Pestic. Manage. Sci. 57 (2001) 958–967.

[35] N. Liu, J.G. Scott, Inheritance of CYT6D1-mediated pyrethroid resistance in house fly (Diptera: Muscidae), J. Econ. Entomol. 90 (1997) 1478–1481.

[36] W.G. Brogdon, J.C. McAllister, J. Vulule, Heme peroxidase activity in single mosquitoes identifies individual expressing an elevated oxidase for insecticide resistance, J. Am. Mosq. Control Assoc. 13 (1997) 233–237.

[37] F.D. Guerrero, R.C. Jamroz, D. Kammlah, S.E. Kunz, Toxicological and molecular characterization of pyrethroid-resistant horn flies, *Haematobia irritans*: identification of *kdr* and *super-kdr* point mutations, Insect Biochem. mol. Biol. 27 (1997) 745–755.

[38] A.C. Kotze, N. Sales, Elevated in vitro monooxygenase activity associated with insecticide resistances in field-

strain larvae of the Australian sheep blowfly (Diptera: Calliphoridae), J. Econ. Entomol. 88 (1995) 782–787.
[39] E. Pisani-Borg, A. Cuany, A. Brun, M. Amichot, D. Fournier, J.B. Berge, Oxidative degradation of diazinon by *Drosophila*—metabolic changes associated with insecticide resistance and induction, Pestic. Biochem. Physiol. 54 (1996) 56–64.
[40] J.A. Wilson, A.G. Clark, N.A. Haak, Effect of piperonyl butoxide on diazinon resistance in field strains of the sheep blowfly, *Lucilia cuprina* (Diptera: Calliphoridae), N Z Bull. Entomol. Res. 89 (1999) 295–301.
[41] C. Sabourault, V.M. Guzov, J.F. Koener, C. Claudianos, F.W. Flapp Jr., R. Feyereisen, Overproduction of a P450 that metabolize diazinon is linked to a loss of function in chromosome 2 ali-esterase (MdaE7) gene in resistant house flies, Insect Mol. Biol. 10 (2001) 609–618.
[42] S-E. Lee, E.M. Lees, Biochemical mechanisms of resistance in strains of *Oryzaephilus surinamensis* (Coleopetera: Silvanidae) resistance to malathion and chlorpyrifos-methyl, J. Econ. Entomol. 94 (2001) 706–713.
[43] A.A Guglielmone, M.E. Castelli, M.M. Volpogni, O.S. Anziani, S.G. Flores, Cypermethrin pour-on synergized with piperonyl butoxide: effects on *Haematobia irritans* (Diptera: Muscidae) natural populations resistant to cypermethrin, Vet. Parasitol. 83 (1999) 65–72.